Development and Validation of an Easy-to-Implement, Practical Algorithm for the Identification of Molecular Subtypes of Gastric Cancer: Prognostic and Therapeutic Implications

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Gastric cancer • Molecular classification • Prognosis • Microsatellite instability • Epithelial-mesenchymal transition

ABSTRACT

Background. Gastric cancer (GC) is a heterogeneous disease, and substantial efforts have been made to develop a molecular biology-based classification system for GC. Analysis of the genomic signature is not always feasible, and thus, we aimed to (i) develop and validate a practical immunohistochemistry (IHC)- and polymerase chain reaction (PCR)-based molecular classification of GC and (ii) to assess HER2 status according to this classification.

Materials and Methods. A total of 894 consecutive patients with GC from two individual cohorts (training, $n = 507$; validation, $n = 387$) were classified using Epstein-Barr virus (EBV) in situ hybridization, microsatellite instability (MSI) testing, and IHC for E-cadherin and p53.

Results. We were able to classify patients into five groups in the training cohort: group 1 (MSI⁺), group 2 (EBV⁻, MSI⁻, non-epithelial-mesenchymal transition [non-EMT]-like, p53⁻),

group 3 (EBV⁺), group 4 (EBV⁻, MSI⁻, non-EMT-like, p53⁺), and group 5 (EBV⁻, MSI⁻, EMT-like). In the training cohort, each group showed different overall survival (OS) after gastrectomy ($p < .001$); group 1 had the best prognosis, and group 5 showed the worst survival outcome. The significant impact of the classification system on OS was also verified in the validation cohort ($p = .004$). HER2 positivity was observed in 6.5% of total population, and most of HER2-positive cases (93.1%) were included in groups 2 and 4.

Conclusion. We developed and validated a modified IHC- and PCR-based molecular classification system in GC, which showed significant impact on survival, irrespective of stage or other clinical variables. We also found close association between HER2 status and non-EMT phenotype in our classification system. *The Oncologist* 2019;24:e1321–e1330

Implications for Practice: Molecular classification of gastric cancer suggested by previous studies mostly relies on extensive genomic data analysis, which is not always available in daily practice. The authors developed a simplified immunohistochemistry- and polymerase chain reaction-based molecular classification of gastric cancer and proved the prognostic significance of this classification, as well as the close association between HER2 status and certain groups of the classification, in the largest consecutive cohort of gastric cancer. Results of this study suggest that this scheme is a cost-effective, easy-to-implement, and feasible way of classifying gastric cancer in daily clinical practice, also serving as a practical tool for aiding therapeutic decisions and predicting prognosis.

INTRODUCTION

Despite recent advances in treatment strategies for gastric cancer (GC) during the last decade [1, 2], GC remains the third most common cause of cancer-related deaths

worldwide according to 2018 global cancer statistics [3]. GC is also recognized for its highly heterogeneous clinicopathologic features [4–6]. Therefore, the need for proper classification of

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GC offering prognostic and therapeutic guidance has long been recognized.

The molecular classification suggested by The Cancer Genome Atlas (TCGA) was a landmark study [7]. It classified GC into four types: Epstein-Barr virus (EBV)-positive type, microsatellite instability (MSI) type, genomically stable (GS) type, and chromosomal instability (CIN) type. Similarly, the Asian Cancer Research Group (ACRG) proposed a classification based on MSI status, epithelial-mesenchymal transition (EMT) signatures by gene expression, and *TP53* mutation signatures [8]. Although enormous amounts of data gathered by extensive molecular genetic experiments were integrated into these classification models, they are barely used in daily clinical practice because of high cost and the time-consuming nature of genomic tests.

The development of practical classification systems for predicting prognosis or treatment response in patients with cancer would be highly useful for clinics. In breast cancer, for example, chemotherapy plans are usually guided by the results of simple ancillary tests, which serve as excellent surrogates representing molecular subtypes of breast cancer: immunohistochemistry (IHC) for hormonal receptors [9] and HER2 protein expression, and additional molecular analysis such as *in situ* hybridization (ISH) testing if necessary [10].

Based on these lessons from breast cancer, efforts have been made to find reliable ways to replace the original genome-based molecular classification with more simplified method-based classification models for patients with GC in routine clinical practice. Setia et al. proposed a classification model based on EBV status, mismatch repair (MMR) protein, and E-cadherin and p53 expression assessed by IHC and showed that their classification had prognostic significance in 146 patients with GC [11]. A similar model was also suggested by Ahn et al., using MLH1 protein expression alone to represent MSI subtype [12]. However, a consensus concerning these alternative classification methods, especially regarding the best IHC method and interpretation criteria, has not been reached. In addition, although *HER2* status remains the only companion diagnostic and treatment predictive factor in GC, the associations between *HER2* status and molecular subgroups of GC are not well studied, partially because of the small number and heterogeneity of *HER2*-positive tumors [4, 13]. More detailed calibration of classification criteria and additional validation of the model using a large population of patients with GC are required for this protein expression-based approach to be adopted into clinical practice.

The aims of this study were as follows: (A) to develop and validate a molecular classification system for GC based on simplified methods such as IHC, which has prognostic significance and can be easily used in clinics, and (B) to assess the association between the modified molecular classification system and *HER2* status.

MATERIALS AND METHODS

Study Population

A total of 894 consecutive patients with GC from two institutions were included; 507 patients were treated in Seoul

National University Bundang Hospital (Seongnam, Republic of Korea) from 2003 to 2005 (training cohort), and the remaining 387 patients were treated in Seoul National University Hospital (Seoul, Republic of Korea) in 2004 (validation cohort). All patients underwent curative or palliative surgical resection, followed by additional adjuvant or palliative chemotherapy if clinically indicated. Clinicopathologic characteristics including overall survival (OS) were retrieved retrospectively from pathology reports and medical records. OS was defined as the time period between the date of surgery and the date of death due to any cause or censoring.

Formalin-fixed, paraffin-embedded tissue blocks from surgically resected samples were obtained from the archives in the Department of Pathology from two institutions. The tissue microarrays (TMAs) were made from the representative 2-mm cores selected by an experienced gastrointestinal pathologist (H.S.L.), as described previously (SuperBioChips Laboratories, Seoul, Republic of Korea) [14].

This study was approved by the institutional review boards (IRBs) of Seoul National University Bundang Hospital and Seoul National University Hospital (IRB numbers: B-1407-260-305 and 1706-105-860).

IHC and EBV ISH

IHC for E-cadherin (clone 36, mouse monoclonal; BD Biosciences, San Jose, CA), p53 (DO7, mouse monoclonal; Dako; Agilent Technologies, Santa Clara, CA), HER2 (4B5; rabbit monoclonal; predilution; Ventana Medical Systems, Tucson, AZ, USA), and MLH1 (BD Biosciences) were performed on 3- μ m-thick slides using an automated immunostainer (BenchMark XT; Ventana Medical Systems), following the manufacturer's guidelines. To assess the EBV status of the study population, we performed EBV ISH with the INFORM EBV-encoded RNA probe (Ventana Medical Systems).

The IHC and EBV ISH were interpreted by two pathologists (J.K. and H.S.L.) who were blinded to the clinical information. The cases showing strong membrane staining of E-cadherin were interpreted as positive expression, whereas altered expression of E-cadherin was defined as either complete loss of membrane staining or an aberrant cytoplasmic staining pattern. For p53, strong nuclear staining in more than 10% of the tumor cells was considered p53 positive, and samples with less than 10% positive cells were interpreted as p53 negative; the cases showing weak, scattered, or patchy positivity were regarded as negative [15, 16]. Tumors showing complete loss of nuclear MLH1 expression were interpreted as loss or aberrant expression [12]. The scoring of HER2 IHC was performed according to Dako HercepTest guidelines for GC as follows [17]: 0, no staining or membrane staining in less than 10% of tumor cells; 1+, faint partial membranous staining in at least 10% of tumor cells; 2+, weak-to-moderate complete or basolateral membranous staining in at least 10% of tumor cells; 3+, moderate-to-strong complete membranous staining in at least 10% of tumor cells (supplemental online Fig. 1A).

MSI Testing

MSI status was determined by a polymerase chain reaction (PCR)-based method using the allele profiles of five markers

(BAT-26, BAT-25, D5S346, D17S250, and S2S123) in tumor cells compared with matched normal samples. PCR products were analyzed by a DNA autosequencer (ABI 3731 Genetic Analyzer; Thermo Fisher Scientific, Waltham, MA). The interpretation of MSI status was performed according to the Revised Bethesda Guidelines [18], briefly as follows: tumors with novel bands in at least two markers were classified as MSI high, tumors with additional alleles in one marker were defined as MSI low, and tumors having identical bands in all five markers were designated as microsatellite stable (MSS).

Bright-Field Dual-Color *HER2* Silver ISH

HER2 silver ISH (SISH) was performed using INFORM *HER2* DNA and INFORM Chromosome 17 (CEP17) probes (Ventana Medical Systems) on an automatic SISH staining device (BenchMark XT, Ventana Medical Systems) according to the manufacturer's guidelines and interpreted as described previously [19]. Briefly, SISH signals were counted in 20 nonoverlapping tumor cell nuclei within the area of hot spot using $\times 60$ objectives by three pathologists (J.K., A.N.S., and H.S.L.) who were blinded to the results of *HER2* IHC. *HER2* amplification was defined as an *HER2*/CEP17 ratio of ≥ 2.0 in 20 tumor nuclei (supplemental online Fig. 1B). For equivocal cases with *HER2*/CEP17 ratio from 0.8 to 2.2, signals from 20 additional tumor nuclei were counted, and *HER2*/CEP17 ratio was recalculated.

The following criteria were applied for determination of the overall *HER2* status: (A) cases showing *HER2* IHC score of 3+ and (B) cases with *HER2* IHC score 2+ and *HER2* amplification by SISH were regarded as *HER2* positive.

IHC- and PCR-Based Molecular Classification of GC

We subdivided the study population into five categories of molecular classification of GC, according to the process adapted from previously reported methods [11, 12]. As shown in Figure 1, EBV⁺ GCs were initially sorted out, and MSI-high GCs were classified as the MSI⁺ group. Among the rest of the MSS or MSI-low GCs, cases showing mesenchymal-like morphologic features or altered E-cadherin expression were classified into the EBV⁻, MSI⁻, EMT-like group. Specifically, mesenchymal-like morphologic features included (A) tumor cells infiltrating into stromal tissue as single cells or (B) elongated tumor cells resembling histiocytes or stromal cells such as myofibroblasts. Determination of mesenchymal-like morphologic features was performed independently by two pathologists (J.K. and H.S.L.). The remaining cases were subdivided according to p53 IHC results; those with positive p53 IHC results were assigned to the EBV⁻, MSI⁻, non-EMT-like, p53⁺ group, and the rest were assigned to the EBV⁻, MSI⁻, non-EMT-like, p53⁻ group. Finally, we named each group as follows: group 1 (MSI⁺), group 2 (EBV⁻, MSI⁻, non-EMT-like, p53⁻), group 3 (EBV⁺), group 4 (EBV⁻, MSI⁻, non-EMT-like, p53⁺), and group 5 (EBV⁻, MSI⁻, EMT-like).

Statistical Analysis

Clinicopathologic characteristics in two cohorts were analyzed by chi square, Fisher's exact, linear-by-linear, and

Kruskal-Wallis tests, as appropriate. Kaplan-Meier analyses of OS according to molecular classification were performed using log-rank tests. Univariable and multivariable analyses were performed by log-rank test and Cox proportional hazard model, respectively. A *p* value less than .05 was considered statistically significant. Statistical analyses were performed using SPSS Statistics 22.0 (IBM, Armonk, NY) and R statistical package 3.4.2 (<http://www.r-project.org>).

RESULTS

Clinicopathologic Characteristics

Clinicopathologic characteristics of the two cohorts are summarized in Table 1. Patients in the validation cohort were relatively younger in terms of age than those in the training cohort (*p* = .008), and there were no significant gender differences between cohorts. The overall distributions of GC subtypes according to World Health Organization (WHO) and Lauren classification were similar between the two cohorts. However, GCs in the validation cohort showed more aggressive pathologic features, including lymphatic invasion (62.5% vs. 49.5%; *p* < .001), venous invasion (18.9% vs. 11.3%; *p* = .001), perineural invasion (50.4% vs. 34.3%; *p* < .001), and advanced stage according to American Joint Committee on Cancer (AJCC) 8th edition (*p* < .001). The validation cohort also showed higher *HER2* IHC scores (*p* < .001) and overall *HER2* positivity (8.5% vs. 4.9%; *p* = .027).

Results of Molecular Classification

We applied the modified molecular classification to the two cohorts separately. Results are summarized in Table 2. In the training cohort, 45 cases out of 507 (8.9%) were in group 1 (MSI⁺), 191 (37.7%) in group 2 (EBV⁻, MSI⁻, non-EMT-like, p53⁻), 51 (10.1%) in group 3 (EBV⁺), 144 (28.4%) in group 4 (EBV⁻, MSI⁻, non-EMT-like, p53⁺), and 76 (15.0%) in group 5 (EBV⁻, MSI⁻, EMT-like). In the validation cohort, the overall distribution of five classes showed no statistically significant differences compared with training cohort (*p* = .071): group 1 (8.3%; 32/387), group 2 (46.0%; 178/387), group 3 (7.2%; 28/387), group 4 (27.6%; 107/387), and group 5 (10.9%; 42/387).

We compared the proportions of different molecular groups categorized by our classification system among different stages (AJCC 8th edition) as shown in supplemental online Table 1 (combined cohort), and significant differences were observed (*p* < .001). The proportion of group 1 cases within stage IV was only 2.6%, which was notably lower compared with other stages (6.8% in stage I, 17.2% in stage II, and 7.5% in stage III). Group 2 showed a predilection toward stage I (54.7%) when compared with other stages (37.3% in stage II, 27.2% in stage III, and 32.1% in stage IV). The proportions of group 3 within each stage were relatively consistent: 9.2% (stage I), 7.7% (stage II), 9.1% (stage III), and 9.0% (stage IV). The distribution of group 4 among the four stages was also relatively even, ranging from 26.0% to 32.1%. However, group 5 showed marked preponderance toward advanced stage: 25.7%

Figure 1. Schematic flow of immunohistochemistry- and polymerase chain reaction-based molecular classification. After EBV⁺ and MSI⁺ gastric cancers (GCs) were sorted out, EMT-like group was determined by morphology and E-cadherin expression by tumor cells. The remaining cases were further classified according to p53 immunohistochemistry results.

Abbreviations: EBV, Epstein-Barr virus; EMT, epithelial-mesenchymal transition; MSI, microsatellite instability.

(88/343) of patients with stage III/IV versus 5.4% (30/551) of patients with stage I/II.

Comparison of Methods for Determination of MSI Status

In order to test whether IHC for MMR protein could serve as the appropriate alternative to PCR-based assessment of MSI status, we performed MLH1 IHC in all available cases (supplemental online Fig. 2). Loss of MLH1 was found in 61 (7.0%) cases out of 874 tested samples. Discordant results between MLH1 IHC and PCR-based study were found in 20 cases (2.3%): 5 cases found to be MSI⁻ by PCR analysis showed loss of nuclear MLH1, and 15 cases classified as MSI⁺ by PCR showed retained expression of MLH1 (supplemental online Table 2). When focusing on cases with MSI⁺ results by PCR, we found that 21.1% (15/71) of these samples showed retained MLH1 expression.

Survival Analysis According to Clinicopathologic Features and Molecular Classification

Survival analyses according to various clinicopathologic features were performed using log-rank tests. EBV status itself was not a significant prognostic indicator (training, $p = .788$; validation, $p = .734$; combined, $p = .791$; supplemental online Fig. 3A), whereas we observed OS differences according to MSI status (supplemental online Fig. 3B), which was statistically significant within combined population (training, $p = .226$; validation, $p = .112$; combined, $p = .045$). There were significant OS differences according to the results of E-cadherin and p53 IHC results ($p < .001$ and $p < .001$, respectively, in combined population; supplemental online Fig. 3C–D). However, no survival difference was observed between *HER2*-negative and *HER2*-positive subgroups (training, $p = .507$; validation, $p = .437$; combined, $p = .668$; supplemental online Fig. 3E).

Table 1. Clinicopathologic features of the study population

Clinicopathologic feature	Training cohort (n = 507), n (%)	Validation cohort (n = 387), n (%)	Combined cohort (n = 894), n (%)	p value ^a
Age				
Median (range), years	62 (25–89)	58 (24–86)	61 (24–89)	.008
< 70 years	400 (78.9)	311 (80.4)	711 (79.5)	
≥ 70 years	107 (21.1)	76 (19.6)	183 (20.5)	
Sex				.175
Male	336 (66.3)	273 (70.5)	609 (68.1)	
Female	171 (33.7)	114 (29.5)	285 (31.9)	
Histology				.768
WDAC	79 (15.7)	38 (9.8)	117 (13.1)	
MDAC	166 (32.9)	137 (35.4)	303 (34.0)	
PDAC	58 (11.5)	58 (15.0)	116 (13.0)	
PCC/SRC	166 (32.9)	134 (34.7)	300 (33.7)	
Mucinous	15 (3.0)	13 (3.4)	28 (3.1)	
GCLS	15 (3.0)	6 (1.6)	21 (2.4)	
Mixed	5 (1.0)	1 (0.3)	6 (0.7)	
Lauren				.226
Intestinal	279 (55.4)	196 (50.6)	475 (53.4)	
Diffuse	221 (43.8)	190 (49.1)	411 (46.1)	
Mixed	4 (0.8)	1 (0.3)	5 (0.6)	
Lymphatic invasion				<.001
Absent	256 (50.5)	145 (37.5)	401 (44.9)	
Present	251 (49.5)	242 (62.5)	493 (55.1)	
Venous invasion				.001
Absent	449 (88.7)	314 (81.1)	763 (85.4)	
Present	57 (11.3)	73 (18.9)	130 (14.6)	
Perineural invasion				<.001
Absent	333 (65.7)	192 (49.6)	525 (58.7)	
Present	174 (34.3)	195 (50.4)	369 (41.3)	
Stage (AJCC 8th edition)				<.001
I	259 (51.1)	123 (31.8)	382 (42.7)	
II	82 (16.2)	87 (22.5)	169 (18.9)	
III	142 (28.0)	123 (31.8)	265 (29.6)	
IV	24 (4.7)	54 (14.0)	78 (8.7)	
HER2 IHC				<.001
Negative/1+	460 (92.0)	246 (68.3)	706 (82.1)	
2+	18 (3.6)	95 (24.5)	113 (12.6)	
3+	22 (4.3)	19 (4.9)	41 (4.6)	
Unknown ^b	7 (1.4)	27 (7.0)	34 (3.8)	
HER2 SISH				.801
Nonamplified	466 (91.9)	313 (80.9)	779 (87.1)	
Amplified	41 (8.1)	30 (7.8)	71 (7.9)	
Unknown ^b	0 (0.0)	44 (11.4)	44 (4.9)	
HER2 status				.027
Negative	482 (95.1)	339 (87.6)	821 (91.8)	
Positive	25 (4.9)	33 (8.5)	58 (6.5)	
Unknown ^b	0 (0.0)	15 (3.9)	15 (1.7)	

^aClinicopathologic features between training cohort and validation cohort were compared.

^bHER2 tests were not conducted because of insufficient tumor cells.

Abbreviations: AJCC, American Joint Committee on Cancer; GCLS, gastric carcinoma with lymphoid stroma; IHC, immunohistochemistry; MDAC, moderately differentiated adenocarcinoma; PCC, poorly cohesive carcinoma; PDAC, poorly differentiated adenocarcinoma; SISH, silver in situ hybridization; SRC, signet ring cell carcinoma; WDAC, well differentiated adenocarcinoma.

Table 2. The results of molecular classification in training and validation cohorts

Molecular classification	Training cohort, n (%)	Validation cohort, n (%)	Combined cohort, n (%)	p value
Group 1: MSI ⁺	45 (8.9)	32 (8.3)	77 (8.6)	.071
Group 2: EBV [−] , MSI [−] , non-EMT-like, p53 [−]	191 (37.7)	178 (46.0)	369 (41.3)	
Group 3: EBV ⁺	51 (10.1)	28 (7.2)	79 (8.8)	
Group 4: EBV [−] , MSI [−] , non-EMT-like, p53 ⁺	144 (28.4)	107 (27.6)	251 (28.1)	
Group 5: EBV [−] , MSI [−] , EMT-like	76 (15.0)	42 (10.9)	118 (13.2)	
Total	507 (56.7)	387 (43.3)	894 (100.0)	

Abbreviation: EBV, Epstein-Barr virus; EMT, epithelial-mesenchymal transition; MSI⁺, microsatellite instability high.

Figure 2. Survival analysis according to molecular classification. In the training cohort (A), groups 1 (microsatellite instability [MSI]⁺) and 2 (Epstein-Barr virus [EBV][−], MSI[−], non-epithelial-mesenchymal transition [non-EMT]-like, p53[−]) showed the best survival outcome, groups 3 (EBV⁺) and 4 (EBV[−], MSI[−], non-EMT-like, p53⁺) showed intermediate prognosis, and group 5 (EBV[−], MSI[−], EMT-like) was noted for poor prognosis ($p < .001$). Similar patterns of differences in overall survival were recapitulated in the validation cohort ($p = .004$) (B) and the combined cohort ($p < .001$) (C). Abbreviation: OS, overall survival.

We also performed survival analyses according to the molecular classification, and Kaplan-Meier survival curves are shown in Figure 2. We found significant OS differences according to the molecular classification in the training cohort ($p < .001$), the validation cohort ($p = .004$), and in the combined population ($p < .001$) as well. In all settings, group 1 (MSI⁺) and group 2 (EBV[−], MSI[−], non-EMT-like, p53[−]) showed the best OS outcome, whereas group 5 (EBV[−], MSI[−], EMT-like) had the worst OS. When OS between subgroups were compared with each other (supplemental online Table 3), group 5 consistently stood out as the poor prognostic indicator in most of the combinations. Univariable analysis on the combined population showed the significant prognostic impacts of advanced age, pathology, advanced stage, and molecular classification ($p < .001$). In addition, we confirmed that the modified molecular classification ($p = .007$) is a statistically significant predictor of OS, independent of other clinical parameters including stage (Table 3).

Distinction Between Signet Ring Cell Carcinoma and Poorly Cohesive Carcinoma

According to the 2010 WHO classification, poorly cohesive carcinomas (PCC), including signet ring cell carcinomas

(SRCs), account for one of the major histologic patterns of GCs. Although WHO classification does not provide distinction between PCC and pure SRCs, our classification was able to discriminate these two histologic subtypes. As shown in supplemental online Table 4, 47.2% (91/193) of PCCs were classified as group 5 (EBV[−], MSI[−], EMT-like), whereas only 8.4% (9/107) of SRCs were in group 3; most of pure SRCs (93/107; 86.9%) fell into group 2 (EBV[−], MSI[−], non-EMT-like, p53[−]) or group 4 (EBV[−], MSI[−], non-EMT-like, p53⁺). In addition to the differences in molecular classification, these two histologic features were also associated with significant difference regarding prognosis: SRCs showed significantly better OS compared with PCCs (supplemental online Fig. 4).

Association Between HER2 Status and Molecular Classification

We assessed HER2 status by performing IHC and SISH, and the results were compared among the groups categorized by our molecular classification system (Table 4). Among 41 patients who were HER2 3+ by IHC, 38 (92.7%) were included in group 2 or 4 ($p = .038$). Similarly, 93.0% (66/71) of the HER2-positive patients by SISH were in group 2 or 4 ($p < .001$). When considering both IHC and SISH results in the determination of HER2 positivity, we noted that a highly

Table 3. Univariable and multivariable analysis of overall survival in the combined cohort ($n = 894$)

	Univariable ^a		Multivariable ^b	
	<i>n</i> (5-yr survival rate)	<i>p</i> value	HR (95% CI)	<i>p</i> value
Age		<.001		
< 70 years	711 (72.6)		1.00	
≥ 70 years	183 (55.7)		2.57 (1.96–3.37)	<.001
Sex		.546		
Male	609 (68.7)		1.00	
Female	285 (70.0)		0.88 (0.68–1.14)	.339
Pathology		<.001		.433
WDAC	117 (90.5)		1.00	
MDAC	303 (65.7)		1.68 (0.90–3.13)	.102
PDAC	116 (67.2)		1.59 (0.80–3.16)	.186
PCC	300 (64.8)		1.72 (0.90–3.30)	.102
Others	55 (69.1)		1.31 (0.61–2.81)	.488
Stage (AJCC 8th edition)		<.001		<.001
I	382 (92.1)		1.00	
II-III	434 (59.8)		6.09 (4.13–8.97)	<.001
IV	78 (7.8)		34.77 (22.15–54.59)	<.001
HER2		.702		.272
Negative	821 (69.1)		1.00	
Positive	58 (66.7)		1.13 (0.71–1.80)	.597
Unknown	15 (80.0)		0.47 (0.17–1.27)	.136
Molecular classification		<.001		.007
Group 1: MSI ⁺	77 (80.5)		1.00	
Group 2: EBV [−] , MSI [−] , non-EMT-like, p53 [−]	369 (76.6)		2.45 (1.44–4.19)	.001
Group 3: EBV ⁺	79 (70.9)		2.47 (1.34–4.58)	.004
Group 4: EBV [−] , MSI [−] , non-EMT-like, p53 ⁺	251 (63.2)		2.59 (1.53–4.37)	<.001
Group 5: EBV [−] , MSI [−] , EMT-like	118 (50.0)		2.89 (1.60–5.23)	<.001

^aIn univariable analysis, Kaplan-Meier method was used (log-rank tests).

^bIn multivariable analysis, Cox proportional hazards model was used.

Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval; EBV, Epstein-Barr virus; EMT, epithelial-mesenchymal transition; HR, hazard ratio; MDAC, moderately differentiated adenocarcinoma; MSI⁺, microsatellite instability high; PCC, poorly cohesive carcinoma; PDAC, poorly differentiated adenocarcinoma; WDAC, well differentiated adenocarcinoma.

significant association exists between *HER2* status and molecular classification ($p < .001$): the majority of *HER2*-positive patients (93.1%; 54/58) were included in either group 2 or group 4.

DISCUSSION

In this study, we developed and validated a modified IHC and PCR-based molecular classification system using two independent, large cohorts of patients with GC. We demonstrated the prognostic impact of this simplified molecular classification in patients with GC and also showed close association between *HER2* status and certain molecular classification subgroups.

Before the era of TCGA, EBV⁺ and MSI⁺ GCs were consistently regarded as the most distinctive subtypes [20–22], and recent efforts focused on how to classify the remaining cases of GCs other than those two types. In addition to EBV⁺ and MSI⁺ GCs, TCGA Research Network proposed GS and CIN subtypes: the former represented by close

association with diffuse type GC by Lauren classification with frequent mutations in *CDH1* and *RHOA* genes, whereas the latter is characterized by genomic instability most notably caused by *TP53* alterations. Although a number of clinicopathologic characteristics, including tumor location, age, and histologic features, showed significant associations with TCGA's molecular classification, survival differences were not found between the four classes [7]. Subsequently, Cristescu et al. suggested another classification based on MSI status, EMT signature, and *TP53* activity, thereby classifying GC into four subtypes, and they found significant differences in survival between the subtypes, which were further validated in additional cohorts of GCs [8].

Despite these improvements with respect to the molecular classification of GC, the impact of this progress on daily diagnostic practice is limited. The most important reason for this discrepancy is that the genome-based molecular tests are too expensive and labor-intensive to be used for clinics. The need for more practical and feasible classification systems that can be easily used in routine clinical

Table 4. Association between HER2 status and molecular classification

	Group 1: MSI ⁺ (n = 77), n (%)	Group 2: EBV ⁻ , MSI ⁻ , non-EMT-like, p53 ⁻ (n = 369), n (%)	Group 3: EBV ⁺ (n = 79), n (%)	Group 4: EBV ⁻ , MSI ⁻ , non-EMT-like, p53 ⁺ (n = 251), n (%)	Group 5: EBV ⁻ , MSI ⁻ , EMT-like (n = 118), n (%)	p value
HER2 IHC						.038 ^a
Negative	36 (46.8)	187 (50.7)	44 (55.7)	131 (52.2)	80 (67.8)	
1+	21 (27.3)	105 (28.5)	21 (26.6)	52 (20.7)	29 (24.6)	
2+	17 (22.1)	41 (11.1)	10 (12.7)	40 (15.9)	5 (4.2)	
3+	1 (1.3)	18 (4.9)	2 (2.5)	20 (8.0)	0 (0.0)	
Unknown ^b	2 (2.6)	18 (4.9)	2 (2.5)	8 (3.2)	4 (3.4)	
HER2 SISH						<.001 ^c
Nonamp	72 (93.5)	321 (87.0)	74 (93.7)	204 (81.3)	108 (91.5)	
Amp	2 (2.6)	33 (8.9)	1 (1.3)	33 (13.1)	2 (1.7)	
Unknown ^b	3 (3.9)	15 (4.1)	4 (5.1)	14 (5.6)	8 (6.8)	
HER2 status						<.001 ^c
Negative	74 (96.1)	337 (91.3)	77 (97.5)	218 (86.9)	115 (97.5)	
Positive	1 (1.3)	27 (7.3)	2 (2.5)	27 (10.8)	1 (0.8)	
Unknown ^b	2 (2.6)	5 (1.4)	0 (0.0)	6 (2.4)	2 (1.7)	

^aLinear-by-linear test was performed.^bHER2 tests were not conducted because of insufficient tumor cells.^cFisher's exact tests were performed.Abbreviations: Amp, amplified; EBV, Epstein-Barr virus; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry; MSI⁺, microsatellite instability high; Nonamp, nonamplified; SISH, silver in situ hybridization.

practice has been increasing. Therefore, studies of IHC-based molecular classification systems as alternative approaches have been conducted, and some results suggest that the IHC-based classification would be easily applicable and have prognostic significance [11, 12, 23, 24].

We have proposed a modified version of a previously described IHC-based molecular classification. First, we adopted PCR-based determination of MSI status rather than the previously used IHC-based method, which determined MSI status by confirming loss of MLH1 expression in GC tumor cells compared with adjacent nonneoplastic cells [11, 12]. Numerous studies have suggested the usefulness and noninferiority of MMR protein IHC as a simpler way of screening for MSI status compared with PCR-based method [25–27]. Using the tissue samples of current study population, we also performed MLH1 IHC and found that the IHC-based method showed highly concordant results compared with the PCR-based assessment. However, it is reported that minority of cases retain intact MLH1 protein expression, although they show obvious MSI phenotype by PCR analyses [28, 29]. Similarly, when confined within MSI⁺ cases determined by PCR in our analysis, we also found a higher discordance rate, suggesting the limited sensitivity of MLH1 IHC. Considering the marked increase in importance of MSI from a treatment related perspective—that MSI tumors show excellent response to immunotherapeutic agents [30]—we think that determination of MSI status should be done by the most sensitive method available, and we thus implemented this PCR-based approach into our classification.

Another key modification of our method is the implementation of histologic characteristics in defining group 5, the EBV⁻, MSI⁻, EMT-like subtype. Previous studies used

E-cadherin IHC as the alternative way to define the group that corresponds to the GS subtype in TCGA's classification [7] or to the MSS/EMT group in the ACRG classification [8]. This idea is largely based on the notion that the E-cadherin protein, coded by the *CDH1* gene, is the key mediator of the EMT process. However, at least in GCs, the correlation between E-cadherin IHC results and *CDH1* gene alteration is not clear [31]; therefore, we thought that defining the EMT feature solely based on E-cadherin IHC would be insufficient. Instead, we suggested adding the histologic criteria of “mesenchymal morphology” into the definition of the EBV⁻, MSI⁻, EMT-like subtype and showed the consistently poor OS of this group compared with others, supporting the clinical usefulness of this approach. Furthermore, the combination of histologic features and immunophenotypic characteristics enabled us to distinguish between pure SRCs and PCC. In addition to proving that these two types with similar but distinct histology were classified differently, we were also able to show significant survival differences between these two.

Most importantly, we succeeded in validating the prognostic significance of this classification system in an independent cohort of patients with GC. To our knowledge, this is the first study in which a molecular classification system for GC using simplified methods that are feasible in real clinical practice setting was validated in an independent large cohort of patients with GC. In addition, we found that this modified molecular classification system is the independent prognostic factor in consecutive GC population by multivariable analysis, irrespective of other clinical factors including stage, further strengthening the prognostic significance of this classification.

In addition to prognostic significance, the major purpose of developing a classification system is for the proper

guidance of the therapeutic approaches. Recent evidence suggests that groups 1 (MSI⁺) and 3 (EBV⁺) may be the ideal candidates for immunotherapy, considering their high expression of PD-L1 protein, numerous numbers of tumor infiltrating immune cells, and higher mutational burdens [32, 33]. Therefore, we suggest that these two groups should be classified by routine tests of EBV and MSI status. The poor prognosis of group 5 patients implies that finding actionable targets in this population is critical. A recent study proposed that the IGF-receptor 1 (IGF1R) inhibitor molecule, linsitinib, showed promising efficacy against mesenchymal phenotype (MP) GCs using cell line-derived xenograft mouse models [34]. Further clarification of the association between MP subtype GCs and group 5 GCs by our classification system should be conducted to set up adequate therapeutic strategies for group 5 GCs. We observed a close association between non-EMT phenotype groups 2 and 4 and HER2 positivity, as reported before [35], and it is well known that HER2-positive cases are the ideal targets for trastuzumab-based therapeutic approach. Still, a more concrete distinction between p53 IHC results and actual *TP53* genetic status would be required for better understanding of molecular biological mechanisms of group 2 and 4 GCs.

Our study has the limitations of being a retrospective study on Asian patients with GC from two different institutions; therefore, for this classification to be more generally accepted for use in clinical practice, additional validation on study populations composed of diverse ethnic backgrounds would be ideal. There is also the possibility that the perception of mesenchymal histology may be different among pathologists; therefore, efforts need to be made to achieve precise definitions and consensus. Construction of TMAs enabled us to validate our classification in the largest cohorts of GC. When constructing TMAs, an experienced pathologist (H.S.L.) reviewed the whole slides of each patient and cautiously selected the most representative tumor areas, and the histologic types of tumors were assigned according the major histologic patterns. During these processes, the nature of mixed carcinoma category of GCs might have been under-represented, and comprehensive characterization of tumor heterogeneity was not ideally performed; how tumor heterogeneity could be reasonably

incorporated into the molecular classification should be the next target of interest in future research.

In conclusion, we have developed a simplified IHC- and PCR-based molecular classification of GC and validated its prognostic implications in the largest consecutive GC cohort ever conducted. We suggest that this classification is an easy-to-implement, practical, prognostically significant method for daily practice as well as a possible guidance tool for making therapeutic decisions.

CONCLUSION

The idea of molecular classification of GC was first introduced by TCGA study; however, the utilization of the classification scheme in daily practice was hindered by the feasibility issue of high-burden molecular genetic studies. We developed a IHC-, ISH-, and PCR-based molecular classification to overcome this feasibility problem, and we showed that this classification has prognostic significance in the largest consecutive GC cohort. We also suggest that this classification can aid making therapeutic decisions and further understanding GC biology.

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DISCLOSURES

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